Programmed cell death in *C. elegans*: the genetic framework

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1. Introduction

Programmed cell death is an important cellular process that controls the development and homeostasis of multicellular organisms, including the nematode *Caenorhabditis elegans.* Genetic and molecular studies in *C. elegans* have led to the identification of many genes that function in different aspects of programmed cell death. These genes define a genetic pathway of programmed cell death in *C. elegans* that is conserved between nematodes and mammals. In this article we will review our current understanding of programmed cell death in *C. elegans* and the insights we learn from functional characterization of the *C. elegans* cell-death genes.

1.1 Advantages of using *C. elegans* for the study of programmed cell death

C. elegans is a free living worm that feeds on bacteria and can be easily maintained in the laboratory (1). The rapid life cycle of *C. elegans* (about 2.5 days at 25^{o}C), the self-fertilizing ability of its hermaphrodite, and its well-defined anatomy have made it an ideal organism for sophisticated genetic manipulations.

C. elegans is transparent; therefore cell divisions and cell deaths can be observed and followed in living animals using high magnification Nomarski optics. This feature has facilitated the determination of the entire cell lineage of *C. elegans* (2-4). Of the 1090 somatic cells that are generated during the development of the *C. elegans* adult hermaphrodite, 131 undergo programmed cell death (2-4). When observed with Nomarski microscopy, cells undergoing programmed cell death adopt a refractile and raised button-like appearance (Figure 1). Each of the 131 deaths occurs at a specific place with its own characteristic time and is essentially invariant from animal to animal. Therefore, mutants with subtle perturbation in the cell death program can be identified and isolated.

Genetic and phenotypic analyses of mutants that are defective in different aspects of programmed cell death have helped define a genetic pathway of programmed cell death in *C. elegans*. The combination of a detailed genetic map, a corresponding physical map (5) and the information provided by the almost finished genome sequencing (only 1% of the *C. elegans* genome left to be determined; 6), further facilitates the molecular studies of programmed cell death in *C. elegans*. Powerful molecular and genetic techniques such as germline transformation (7) and genetic mosaic analysis (8) have been developed. These techniques in combination with detailed knowledge of the anatomy and cell lineage of *C. elegans* have catalyzed rapid progress in our understanding of the mechanisms of programmed cell death.

1.2 Origin and identity of dying cells in *C. elegans*

Most (113/131) developmental cell deaths of a hermaphrodite occur during embryogenesis, many of which during a short period of time between 250 and 450 minutes after fertilization (4). The remainder occurs during early larval development (2). In males a few more cell deaths are observed in male-specific lineage and during late larval stages. In adult animals, no more somatic cell deaths are observed in either sex.

However, a large number of cells in the germline of hermaphrodites, but not those of males, undergo programmed cell death (9).

Programmed cell death can be viewed as a terminal differentiation fate. The presumptive types of cells programmed to die have been inferred mainly from their nuclear morphology before the death process, their lineally equivalent homologs that live, and more directly, from the cell fates they adopt when they survive in those mutants that lack programmed cell deaths. It appears that most of the cell deaths in *C. elegans* are neuronal deaths: 105/131 and 117/147 cells that die are neurons in hermaphrodites and males, respectively. In addition to neurons, many other types of cells including neuron-associated cells, hypodermal cells, muscle cells, and pharyngeal gland cells, undergo programmed cell death (2, 4). No intestinal cells die in *C. elegans*, although deaths of intestinal cells have been observed in other nematode species (10).

Programmed cell deaths are not confined to a specific cell lineage during *C. elegans* development. Rather, they are asymmetrically distributed among cell lineages (Figure 2). Deaths are found in descendants of three primary blastomeres (AB, MS and C), most (116/131) from the AB lineage that produces most of the nervous system. Cell death appears to be a common fate in the AB lineage in which 116 of the 722 cells generated proceed to die, presumably a reflection of many dying neurons. No cell death is found in the E lineage, which generates only intestine, or the D lineage, which generates only muscle. The germline produced from the P4 lineage gives rise to variable number of cell deaths in hermaphrodite adults (9).

1.3 Roles of programmed cell death in *C. elegans*

As in other organisms, programmed cell death in *C. elegans* plays important roles in different aspects of nematode development. Programmed cell death eliminates cells that are generated in extra or cells that have already fulfilled their functions and are no longer needed. For example, the linker cell of the male gonad is generated in the second larval stage and guides the extension of the gonad during development (3). Once extension of the gonad is completed at the fourth larval stage, the linker cell is no longer needed and undergoes programmed cell death. Programmed cell death is also important for generating sexual dimorphism in *C. elegans*. For example, cells specific to one sex die during the development of the opposite sex (4). In addition, cell deaths can be used in the sculpturing process to give rise to species-specific organs and body structures. For example, two distal tip cells in *C. elegans* guide the extension of the anterior and posterior ends of the developing gonads and lead to two symmetric gonadal arms. Laser ablation of the posterior distal tip cell leads to a cessation of growth of the posterior gonadal arm (11). In another nematode species *P. redivivus*, the programmed death of the posterior distal tip cell is responsible for the development of one-armed gonad in females (12).

1.4 Morphology and kinetics of programmed cell death in *C. elegans*

The morphological changes of cells undergoing programmed cell death have been characterized using Nomarski optics and electron microscopy (2, 13). The cell doomed to die is often smaller than its sister cell

right after its birth. When viewed with Nomarski optics, the dying cell first shows lessened refractility in the cytoplasm which is followed by decreased refractility in the nucleus. Soon after that, both cytoplasm and nucleus become highly refractile, adopting a raised and flattened button-like appearance (Figure 1). The mechanism underlining the refractility change during the death process is not well understood. Eventually, the refractile cell corpse disappears since it is engulfed and digested by its neighboring cell. The entire process of cell death from the birth to the disappearance of the cell occurs approximately within an hour (Figure 1; 2, 14).

At the ultrastructural level, as a cell undergoes programmed cell death the cytoplasm condenses and the nuclear chromatin aggregates. The engulfment process appears to occur in the very early stage of cell death: membrane processes extending from the neighboring engulfing cell can be found even before the dying cell displays any visible morphological change (13). During the mid-stage of the cell death process, the body of the dead cell is split into several membrane-bound fragments by phagocytotic arms of the engulfing cell. Internal membranes and plasma membranes of the dead cell adopt a whorled appearance and often are enclosed within autophagic vacuoles. At the final stage, portions of the fragmented cell body fuse with vacuoles inside the engulfing cell (13).

C. elegans programmed cell death shares features with those cell deaths observed in both invertebrates and vertebrates termed apoptosis (15, 16). Both cell deaths show similar characteristic morphological changes at the ultrastructural level, such as cytoplasm condensation, chromatin aggregation and engulfment of dead cells by engulfing cells. Recently, DNA fragmentation has also been detected during *C. elegans* programmed cell death as assayed using the TUNEL (TdT-mediated dUTP nick-end labeling) technique (Y.C. Wu, G.M.Stanfield and H.R. Horvitz, personal communication). However, the formation of the nucleosomal DNA ladder, a hallmark of apoptosis, has yet to be demonstrated in *C. elegans* programmed cell death.

2. Genetic and molecular analysis of programmed cell death in *C. elegans*

2.1 The genetic pathway of programmed cell death in *C. elegans*

Over the past twenty years, genetic studies in *C. elegans* have led to the identification of more than a dozen genes that are involved in different aspects of programmed cell death. Three genes, *nuc-1* (nuclease-deficient), *ced-1* and *ced-2* (cell-death abnormal), were first identified as genes that are involved in the removal or degradation of cell corpses: mutations in the *nuc-1* gene block degradation of DNA from dead cells; mutations in *ced-1* or *ced-2* gene prevent the engulfment of many cell corpses, leading to the mutant phenotype of persistent cell corpses (Figure 3; 14, 17). Subsequently, mutations in two additional genes, *ced-3* and *ced-4*, were isolated. Mutations in *ced-3* were isolated as suppressors of the *ced-1* mutants, whereas the first allele of *ced-4* was identified as a suppressor of an egg-laying defective mutant in which two hermaphrodite specific motor neurons (HSNs) important for hermaphrodite egg-laying control inappropriately undergo programmed cell

death (18). Strikingly, mutations in both *ced-3* and *ced-4* genes prevent most, if not all, programmed cell death in *C. elegans*, suggesting that both genes are required for the execution of cell death. Mosaic analysis of *ced-3* and *ced-4* mutants suggest that both genes act within dying cells to cause cell death (19). This observation provides the first genetic evidence that cells die by an intrinsic suicide mechanism. Soon after that, more cell-death genes were identified in various genetic screens for new mutations that affect different aspects of programmed cell death. These include four additional genes that mediate cell-corpse engulfment (*ced-5*, *ced-6*, *ced-7*, and *ced-10;* 20), two genes (*ces-1* and *ces-2;* cell death specification) that control the death fate of a specific set of cells (21), the *ced-9* gene that generally protects cells from programmed cell death (22), and finally, the *egl-1* gene that is also required for almost all programmed cell deaths (23).

The *ced-9* gene was initially identified by a gain-of-function (*gf*) mutation that prevents most programmed cell deaths in *C. elegans* as mutations in *ced-3* or *ced-4* do. The normal function of *ced-9* was revealed by the phenotype of its loss-of-function (*lf*) mutants in which many cells that would normally live undergo programmed cell death, suggesting that *ced-9* acts to protect cells from programmed cell death (22). The *egl-1* gene was originally defined by several gain-of-function mutations that cause inappropriate death of HSN neurons in hermaphrodites (24). Subsequent isolation of an *egl-1* loss-of-function mutation as a *cis*-dominant suppressor of *egl-1*(*gf*) mutations and examination of the *egl-1*(*lf*) phenotype suggest that the *egl-1* gene is required for almost all programmed cell deaths rather than just playing a role in specifying the death of HSN neurons as its *gf* mutant phenotype implicates (23).

Genetic epistasis analyses and phenotypic analyses of the above cell-death mutants have placed these cell-death genes into a genetic pathway that contains four sequential and genetically separable steps of cell death: the decision making step of which cell should die, the killing process of cell death, the engulfment of cell corpses, and the degradation of cellular debris (Figure 4; 25). In the following section, we will review current status of the genetic and molecular characterization of genes in this pathway.

2.2. Genes involved in the killing process of programmed cell death

(1) CED-3 – The death protease that executes programmed cell death

The *ced-3* gene is essential for cells to die by programmed cell death in *C. elegans*. In strong *ced-3* loss-of-function mutants, almost all programmed cell deaths fail to occur (18). The undead cells in *ced-3* mutants, though never divide, can differentiate to adopt a cell fate that is very similar to that of their sister cell or a cell fate similar to that of their aunt or of cells at equivalent positions in a related cell lineage (18). Some of the undead cells can even function under special circumstances (26). Intriguingly, unlike in other organisms, the large number of extra undead cells in *ced-3* mutant animals, constituting 12% more somatic cells, do not seem to severely interfere with the functions of

normal cells, since *ced-3* mutants are superficially indistinguishable from wild-type animals and do not show obvious morphological or behavioral abnormalities (18). However, subtle defects such as slow growth, slightly reduced brood size, and impaired chemotaxis response have been observed with *ced-3* mutant animals (25, 27).

Genetic mosaic analysis of *ced-3* suggests that *ced-3* acts within dying cells to cause programmed cell death (19). *ced-3* encodes a protein with significant sequence similarity to a family of cysteine proteases named caspases (cysteine aspartate-specific protease), which cleave their substrates exclusively after an aspartate amino acid (28, 29). Several caspases have been shown to mediate apoptosis in other organisms (30-34), suggesting that the caspases and CED-3 may define a new family of proteins important for the execution of programmed cell death in many species. Like many other caspases, CED-3 is initially synthesized as a 56 kD proenzyme and can be proteolytically activated to generate an active cysteine protease that is composed of p17/p13 protease subunits (29, 35). The active CED-3 protease has substrate specificity similar to that of mammalian caspase-3 (35). Several mutations that severely reduce *ced-3* killing activity in nematodes affect the residues that are conserved among many caspases and have been implicated to be critical for the catalytic activity of the proteases (28, 36-39). Furthermore, analysis of *in vitro* protease activities from several mutant CED-3 proteins demonstrated that the extents of reduction of CED-3 protease activities correlate directly with the extents of reduction of *ced-3 in vivo* killing activities (35). These observations indicate that the CED-3 protease activity is essential for *ced-3* to cause programmed cell death in *C. elegans*.

The regulation of *ced-3* killing activity so that CED-3 is activated in the right cells and at the right times has been intensively investigated. The *ced-3* gene appears to be expressed in most, if not all, cells in *C. elegans*, since mutations in *ced-3* can block the ectopic deaths of many cells from different cell types caused by *ced-9*(*lf*) mutations (22). This observation argues that *ced-3* is expressed in many cells so that it can mediate deaths of these normally living cells in *ced-9(lf)* mutants and that *ced-3* activity is inhibited by *ced-9* in living cells. Overexpression of *ced-3* in *C. elegans* can result in constitutive activation of *ced-3* activity and lead to the death of the cells where *ced-3* is ectopically expressed (40). This result and the observation that the CED-3 proenzyme can be proteolytically activated when overexpressed in bacteria suggest that the activation of *ced-3* can be achieved by increased expression or concentration of the CED-3 proenzyme (35). The expression patterns of *ced-3* have not been determined *in C. elegans*. It is not clear whether the expression of *ced-3* is increased in cells that are fated to die.

Oligomerization of caspase zymogens that are linked to trimerized surface receptors induced by ligand binding or other oligomerized protein complexes has been shown to be important for the activation of mammalian procaspases such as procaspase-8 and procaspase-9, presumably by bringing the protease domains of these caspase zymogens to proximity and facilitating inter-

molecular proteolytic cleavage (41-44). Similarly, induced oligomerization of CED-3 protease domains can also result in the processing of CED-3 into mature protease subunits (p15/p17) *in vitro* and the activation of CED-3 killing activity in cell culture (45). Increased concentration of CED-3 in cells may enhance the chance of CED-3 oligomerization and thus the chance of its activation. It has also been postulated that oligomerization of CED-3 can be achieved by a second mechanism: the oligomerization of another essential cell-death protein CED-4 (45, 46). CED-4 has been shown to interact with CED-3 and can self-oligomerize *in vitro*. Mutant CED-4 proteins that can not oligomerize fail to activate CED-3 killing activity in mammalian cells, suggesting that CED-4 oligomerization may be an important part of the CED-3 activation process (45).

The activity of *ced-3* can also be controlled by negative regulators. The baculovirus p35 protein that is required to block baculovirus-infected insect cells from apoptosis has been shown to be able to inhibit cell death in diverse organisms including *C. elegans* (47-51). These observations suggest that p35 may affect an evolutionarily conserved component in the cell death pathway. Biochemical and molecular genetic studies suggest that p35 appears to do so by directly inhibiting the protease activity of CED-3 or other death caspases (52, 53). In *C. elegans*, no sequence homolog of p35 has been identified so far. However, the general cell-death inhibitor CED-9 may be able to play a similar role. CED-9 has been found to be a substrate of the CED-3 protease *in vitro* (54). Mutations that disrupt CED-3 cleavage sites in CED-9 significantly reduce the death-protective activity of CED-9 in *C. elegans*, suggesting that CED-9 may function directly as a substrate inhibitor of the CED-3 death protease (54).

After CED-3 is activated, it may cause cell death by cleaving a few critical substrates. Cleavage of CED-3 targets may activate some death-promoting activities and/or inactivate death-inhibiting regulators and thus lead to systematic cell disassembly and the eventual recognition and engulfment of the cell corpse by its neighboring cell. So far no genes that act downstream of *ced-3* in the genetic pathway have been found to encode a substrate of the CED-3 protease.

(2) CED-4 – The regulator of CED-3 activation

Like the *ced-3* gene, the activity of *ced-4* is also required cell-autonomously for cells to die by programmed cell death (19). *ced-4* encodes a protein that is similar to mammalian Apaf-1 (Apoptotic protease activating factor 1), a factor that is critical for the activation of mammalian caspases involved in apoptosis (46, 55-57). By analogy, *ced-4* may play a similar role in activating the activity of *ced-3*. From northern blot analysis, *ced-4* transcript is found primarily in the embryo stage when most programmed cell deaths (113 out of 131 in hermaphrodites) occur (46). *ced-4* expression is not altered by mutations in the *ced-3* gene, suggesting that *ced-3* does not regulate the expression of *ced-4*. Like *ced-3*, *ced-4* is likely to be expressed in many cells, since loss-of-

function mutations in *ced-4* also block the ectopic deaths of many cells caused by *ced-9*(*lf*) mutations (22).

Overexpression of *ced-4* in *C. elegans* can result in deaths of cells where *ced-4* is ectopically expressed (40). However, this cell killing caused by overexpression of *ced-4* is not very efficient. The killing efficiency is greatly increased if the endogenous *ced-9* activity is eliminated by *ced-9*(*lf*) mutations but markedly reduced in *ced-3*(*lf*) mutants (40). This observation and the finding that *ced-4*(*lf*) mutations fully block ectopic cell deaths in *ced-9*(*lf*) mutants indicate that *ced-4* acts genetically downstream of or in parallel to *ced-9* but upstream of or in parallel to *ced-3* (Figure 4). Consistent with this genetic ordering, cell killing mediated by overexpression of *ced-3* is not obviously affected in the absence of the endogenous *ced-4* activity (40).

Biochemical studies of CED-3, CED-4 and CED-9 have provided important insights into how these three proteins may interact with one another to regulate cell death. CED-4 has been found to physically interact with both CED-3 and CED-9 *in vitro* and can enhance the activation of the *ced-3* killing activity in cultured cells when co-transfected with *ced-3*, which can be suppressed by co-transfection additionally with *ced-9* (58-62). The binding of CED-9 and CED-3 to CED-4 is not mutually exclusive (45, 59). This leads to the hypothesis that CED-3, CED-4 and CED-9 in living cells may co-exist as a ternary protein complex in which CED-3 remains an inactive proenzyme (Figure 5 A). In cells that are doomed to die, certain cell death factors or signals will trigger the release of the CED-4/CED-3 complex from CED-9 and the subsequent oligomerization of CED-4 will bring CED-3 proenzymes to proximity and lead to its autoproteolytic activation (Figure 5; 45, 59, 63, 64). It is also possible that CED-3 does not associate with CED-4/CED-9 complex and exists as monomer in cytosol. In this case, the release of CED-4 from CED-9 would allow for the formation of a CED-3/CED-4 complex and the subsequent CED-3 activation. Studies of the subcellular localization patterns of three proteins in *C. elegans* cells should be able to address this issue.

CED-4 and Apaf-1 share sequence similarity over a stretch of 320 amino acids which contain Walker's A- and B- motifs, both of which are indicative of binding and hydrolysis of a nucleotide triphosphate (46, 55). Apaf-1 *in vitro* needs the presence of both cytochrome c and dATP (or ATP but not any other nucleotide) to catalyze the processing of procaspase-9 (55, 65, 66). Furthermore, Apaf-1 activity is potently inhibited by ATPγS (a nonhydrolyzable ATP analogue; 66), suggesting that an ATPase activity may be required for Apaf-1 to function. Indeed, purified recombinant Apaf-1 can bind and hydrolyze ATP or dATP to ADP or dADP, respectively. The hydrolysis of ATP/dATP and the binding of cytochrome c promote Apaf-1 oligomerization and the subsequent recruitment and activation of procaspase-9 (67). The importance of the nucleotide-binding motifs is underscored by the experiments in which mutations in the conserved residues of two Walker's motifs abolished the activity of CED-4 or Apaf-1 in activating their respective procaspases (59, 61,

67). Interestingly, one of the Walker's A-motif mutations (K165R) which inactivates CED-4 does not seem to affect the ability of CED-4 to oligomerize, indicating that oligomerization of CED-4 is not sufficient to activate CED-3 (45). So far, no ATPase activity has been demonstrated for CED-4, although CED-4 has been shown to bind ATP *in vitro* (68). The role of the putative ATPase activities of CED-4 and Apaf-1 in catalyzing procaspase activation is not clear and is currently under investigation.

The regulation of *ced-4* activity is also important for the appropriate control of programmed cell death in nematodes. Alternative splicing of *ced-4* transcripts has been implicated for such regulation. Specifically, *ced-4* was found to encode two transcripts: the major and the shorter one (*ced-4S* transcript) causes programmed cell death; whereas the minor and the longer one (*ced-4L* transcript) can act to prevent programmed cell death (69). CED-4L contains a twenty-four amino acid insertion right after amino acids 213 of CED-4S between the two Walker's motifs. Despite of this small insertion, CED-4L can self-oligomerizes as well as CED-4S and shows similar binding affinity to CED-3 and CED-9 as CED-4S (45, 59). It is not clear whether this insertion disrupts the ATP binding ability or putative ATPase activity of CED-4S. If it does, then CED-4L may simply act as a dominant-negative form of CED-4S to interfere with CED-4S' function. CED-4 activity may also be regulated by other factors. Recently, *C. elegans* protein MAC-1, a member of AAA family of ATPase, has been shown to bind CED-4 (70). Overexpression of MAC-1 in *C. elegans* can prevent some natural cell deaths in a sensitized genetic background, suggesting that MAC-1 may antagonize or negatively regulate the activity of CED-4 (70).

(3) CED-9 – The protector against programmed cell death

The *ced-9* gene was first defined by a gain-of-function mutation (*n1950)* that prevents almost all programmed cell deaths in *C. elegans* as do mutations in the *ced-3* or *ced-4* gene (22). *ced-9* loss-of-function mutations were isolated as *cis*-dominant suppressors of this *gf* allele. *ced-9*(*lf*) mutants have the opposite phenotype to that of the *ced-9*(*gf*) mutant in that many cells that normally live undergo ectopic cell deaths (22). Thus *ced-9* acts as a negative regulator of programmed cell death. The extent of ectopic deaths in *ced-9*(*lf*) mutants is dependent on the maternal *ced-9* genotype of these animals. Homozygous *ced-9*(*lf*) animals derived from *ced-9*(*lf*)/+ mothers have much fewer cell deaths than those derived from homozygous *ced-9*(*lf*) mothers and usually can survive into adulthood, presumably because the maternal *ced-9* product is sufficient to prevent most cells from cell death. By contrast, homozygous *ced-9*(*lf*) animals derived from homozygous *ced-9*(*lf*) mothers invariably have a large number of ectopic cell deaths and die in early embryonic stage, leading to the maternal lethality phenotype (22). As mentioned above, the phenotypes of massive ectopic cell deaths and embryonic lethality caused by *ced-9*(*lf*) mutations can be fully suppressed by strong *lf* mutations in either *ced-3* or *ced-4* gene, indicating that *ced-9* acts upstream of both *ced-*

3 and *ced-4* to negatively regulate their cell-killing activities. In addition, *ced-9*(*lf*); *ced-3*(*lf*) animals or *ced-4*(*lf*) *ced-9*(*lf*) animals do not exhibit any other obvious defects beyond those observed in *ced-3*(*lf*) or *ced-4*(*lf*) single mutants, suggesting that *ced-9* may only have a role in regulating programmed cell death in *C. elegans* (22).

The product of the *ced-9* gene shares significant sequence homology with that of human proto-oncogene *bcl-2* (23% identity), which plays a similar role in preventing programmed cell death in mammals (71-76). In B-cell lymphoma, the *bcl-2* locus is translocated from chromosome 14 to chromosome 18 and fused to the immunoglobulin heavy chain gene, resulting in overexpression of the *bcl-2* gene and suppression of natural programmed cell deaths in B cells (72-76). Interestingly, overexpression of *bcl-2* can also inhibit programmed cell death in nematodes and can even substitute for *ced-9* to prevent ectopic cell deaths in *ced-9*(*lf*) animals (54, 71, 77). These results strongly suggest that *ced-9* and *bcl-2* not only have sequence similarity but also have functional similarity and that the cell death pathway is conserved between nematodes and mammals.

ced-9 and *bcl-2* are two members of a rapidly growing gene family that play important roles in regulating programmed cell death in diverse species (for review see ref 78). Members of this gene family can either be pro-apoptotic or anti-apoptotic. Some members like Bcl-x and Bcl-2 can even produce both pro- and anti-apoptotic variants (79, 80). All members contain at least one Bcl-2 homology region (BH domain) and some members including CED-9 and Bcl-2 contain up to four BH domains (78). Both CED-9 and Bcl-2 have a hydrophobic tail that may be important for targeting these proteins to various membrane organelles such as mitochondria, nuclear membrane, and endoplasmic reticulum, as have been shown in Bcl-2 (81, 82). Two *ced-9*(*lf*) alleles, *n2812* and *n2077*, have nonsense mutations at codon positions 46 and 160, presumably producing truncated CED-9 proteins (71). The third *ced-9*(*lf*) allele, *n1653*, which causes the substitution of tyrosine 149 to glutamine, is temperature-sensitive. Intriguingly, the *ced-9(gf)* mutation, *n1950*, does not seem to cause overexpression of *ced-9* but rather a single amino acid change of a highly conserved residue in the BH1 domain of CED-9 (glycine 169 to glutamate; 83). How this *gf* mutation results in increased *ced-9* protective activity remains to be investigated.

Interestingly, as in the cases of Bcl-xL and Bcl-2, *ced-9* also appears to generate both death-promoting and death-preventing activities (83). This conclusion came from two observations. First, the wild-type *ced-9* activity seems to be able to attenuate the death preventing activity provided by *ced-9*(*gf*) mutation, since *ced-9*(gf)/+ animals have less surviving cells than *ced-9(gf)/Df* animals (*Df*: deficiency; 83). Second, *ced-9* seems to promote the deaths of those cells that normally die. In a weak *ced-3* mutant background which has fewer extra undead cells than strong *ced-3* mutants, *ced-9*(*lf*) mutations further increase the number of undead cells, an observation more consistent with the idea that *ced-9* promotes killing rather than surviving in those cells that die (83).

Given that *ced-9*(*lf*) mutations cause many ectopic cell deaths, *ced-9* must be expressed in most of the living cells to protect them from deaths. Overexpression of *ced-9* in nematodes can prevent most of the programmed cell deaths, suggesting that the level of *ced-9* expression is critical in determining whether a cell lives or dies. Interestingly, *ced-9* is transcribed as a polycistronic message with another gene *cyt-1* that encodes a protein similar to cytochrome b_{560} of complex II of the mitochondria respiratory chain (71). The ORF (open reading frame) of the *cyt-1* gene does not seem to be required for the *ced-9* function, since a transgene containing the *ced-9* region but not most of the *cyt-1* region can still rescue *ced-9*(*lf*) mutants efficiently (71). However, the recent findings that components of mitochondria such as cytochrome c and AIF (apoptosis inducing factor) are actively involved in the activation of the death program in mammalian cells (65, 84) and that CED-9 is associated with mitochondria in mammalian cells (60) have made this co-transcription observation rather intriguing.

In vitro, CED-9 directly interacts with CED-4 and its upstream regulator EGL-1 (23, 58-60, 85). In mammalian cells CED-9 and CED-4 have been found to be co-localized to mitochondria (60). The interaction of CED-9 with CED-4 has been postulated as a mechanism to prevent CED-4 from activating CED-3 proenzyme (Figure 5). *ced-9(lf)* mutation *n1653* reduces the interaction between CED-4 and CED-9, which is consistent with the hypothesis that CED-9 inhibits CED-4 activity through protein interaction (58, 61). CED-9 also interacts with EGL-1 and the binding of EGL-1 to CED-9 can effectively release CED-4 from CED-4/CED-9 complex, therefore allowing CED-4 to activate CED-3 (23, 85). Furthermore, CED-9 can directly interact with CED-3 *in vitro* and is an excellent substrate of the CED-3 protease (54). CED-3 can cleave CED-9 at two sites (aspartate 44 and aspartate 67). While mutations at either cleavage site do not affect CED-9 death protective activity in nematodes, mutations that disrupt both CED-3 cleavage sites in CED-9 markedly reduce the protective activity of CED-9, suggesting that the presence of at least one CED-3 cleavage site is important for CED-9 death protective function (54). Cleavage of CED-9 by CED-3 generates a carboxyl-terminal product that resembles Bcl-2 in sequence and that has similar death protective activity as Bcl-2 in *C. elegans*. This carboxyl-terminal region of CED-9 is also sufficient to mediate interaction with CED-4 (D. Xue and H.R. Horvitz, unpublished results). Furthermore, a chimeric protein containing the amino-terminal region of CED-9 (amino acids 1-80) including two CED-3 cleavage sites and the entire Bcl-2 protein protects against programmed cell death in *C. elegans* significantly better than the Bcl-2 protein alone (54). These results indicate that CED-9 may inhibit cell death in *C. elegans* through two distinct mechanisms. First, CED-9 may directly inhibit the activity of the CED-3 death protease through its two CED-3 cleavage sites, maybe acting as a competitive substrate inhibitor. Second, CED-9 may indirectly inhibit the activation of CED-3 by forming a complex with CED-4 through its carboxyl-terminal Bcl-2 homology regions.

(4) EGL-1 – The initiator of programmed cell death

The discovery that the *egl-1* gene is required for the deaths of many cells in *C. elegans* came as a pleasant surprise when a loss-of-function intragenic suppressor (*n3082*) of an *egl-1*(*gf*) mutation (*n1084*) was found to result in survival of almost all the cells that normally die (23). *egl-1* has long been regarded as a gene that specifies the death of HSN neurons since all *egl-1*(*gf*) mutations identified appear to cause no other defect except for the ectopic death of two HSN neurons in hermaphrodites (24, 86). The death of HSN neurons in *egl-1*(*gf*) mutants can be suppressed by *ced-9*(*n1950, gf*), *ced-3*(*lf*), or *ced-4*(*lf*) mutations, indicating that *ced-9, ced-3* and *ced-4* acts downstream of or in parallel to *egl-1* (18, 22, 24). Overexpression of *egl-1* can cause many cells to undergo ectopic cell deaths, suggesting that *egl-1* like *ced-3* and *ced-4* encodes a cell-killing activity (23). The ectopic cell deaths caused by overexpression of *egl-1* can also be suppressed by *ced-9*(*gf*), *ced-3*(*lf*), or *ced-4*(*lf*) mutations. In contrast, the *egl-1*(*lf*) mutation can not suppress ectopic cell deaths caused by *ced-9*(*lf*) mutations (23). These results further support the conclusion that *egl-1* acts upstream of the *ced-3*, *ced-4* and *ced-9* genes. Genetic epistasis analysis on *egl-1* and cell-death-specification genes *ces-1* and *ces-2* suggests that *egl-1* is likely to act downstream of these death specification genes. Since *egl-1* is the earliest acting gene of the general cell-death pathway identified so far and acts downstream of the death specification genes, *egl-1* may play a crucial role in receiving cell death signals from the cell-death-specification genes and then initiating the activation of the cell death program.

egl-1 encodes a relatively small protein of ninety-one amino acids that has no sequence similarity to any other protein except a nine amino-acid stretch that resembles the BH3 motif of the Bcl-2 protein family (23). The BH3 domain in several Bcl-2 family proteins, especially in those BH3-only subfamily proteins including Bik, Bid, Harakiri, and Bim, has been shown to be important for inducing apoptosis by binding to anti-apoptotic Bcl-2 family proteins such as Bcl-xL and Bcl-2 and antagonizing the activity of these anti-apoptotic proteins (for review see ref 78). Similarly, EGL-1 *in vitro* binds CED-9 in a BH3-domain-dependent manner (23, 85). Furthermore, the binding of EGL-1 to CED-9 inhibits the interaction of CED-4 with CED-9 and may thus set CED-4 free to activate CED-3 (Figure 5; 85). *n3082*, the only loss-of-function allele of *egl-1* identified so far, has a five nucleotide deletion in exon 2 that results in the formation of a truncated EGL-1 without the BH3 domain and the carboxyl terminus of the protein. This truncated version of EGL-1 fails to interact with CED-9 *in vitro*, providing further evidence that the interaction between EGL-1 and CED-9 is important for EGL-1 death-inducing activity *in vivo* (23). The nature of the *egl-1(gf)* mutations and the expression patterns of the *egl-1* gene have not been reported.

(5) Others

There are a few other genes that have not been characterized in detail but have been shown to be important for programmed cell death in *C. elegans*. The *ced-8* gene was first identified as a gene important for the engulfment of cell-corpses, since mutations in *ced-8* result in a significant increase of the number of cell corpses in late-stage embryos and young larvae in which few cell corpses is seen in wild-type animals. This phenotype is reminiscent of the engulfment-defective mutant phenotype (20). However, time course analysis of the cell corpse numbers at different embryonic stages of the *ced-8* mutant animals revealed that the increased number of cell corpses in late-stage embryos was due to the delay of the appearance of dying cells from early-stage embryos to the late-stage ones (G.M. Stanfield and H.R. Horvitz, personal communication; 27). *ced-8* may thus play a role in regulating the kinetics of cell killing in *C. elegans*.

The *dad-1* gene (defender against apopototic death) is another gene that has been implicated in regulating programmed cell death in *C. elegans* (87). The mammalian *dad-1* gene was originally identified as a gene that could rescue the apoptotic phenotype of a temperature-sensitive mutant hamster cell line at the restrictive temperature (88). The *C.elegans dad-1* gene was identified based on its sequence homology with mammalian *dad-1* (>60% identity). Importantly, overexpression of *C. elegans* or human *dad-1* gene can partially inhibit programmed cell death in *C. elegans* (87), suggesting that *dad-1* may function as a negative cell-death regulator in *C. elegans*. Interestingly, *dad-1* shares significant sequence similarity (40% identity) with a yeast gene OST2 that encodes the 16-kD subunit of the yeast oligosaccharyltransferase, an enzyme complex that catalyzes glycosylation of newly synthesized proteins in the lumen of the rough endoplasmic reticulum (89). The implication of this sequence similarity, the target(s) of *dad-1*, and the mechanism by which *C. elegans dad-1* inhibits cell death remain to be determined. So far, no genetic mutation has been identified in the *C. elegans dad-1* gene.

2.3. Genes involved in the specification of the cell-death fate in specific cell types

In *C. elegans*, all cell deaths have been "preprogrammed" –- the cells that die and the times when they die are invariant from animal to animal (2-4). Thus the activation of the cell death program must be tightly controlled so that cell deaths occur in the right cells and at the right times. Genetic studies in *C.elegans* suggest that the life vs. death decisions of individual cells are likely to be regulated by cell-type specific regulatory genes (21). These regulatory genes may control cell deaths by modulating the activities or the expression of key components in the central cell-killing pathway. In the following we review what we have learned about the control of cell deaths in some specific cell types.

(1) Sister cells of NSM neurons

Two genes, *ces-1* and *ces-2* (cell death specification), have been found to regulate the initiation of cell death in two cells of *C. elegans*, the sister cells of the pharyngeal serotonergic NSM neurons. In wild-type animals, the NSM sister cells undergo programmed cell death. However, a gain-of-function mutation (*n703*) in the *ces-1* gene or a reduction-of-function mutation (*n732*) in the *ces-2* gene can cause survival of these two cells (21). These two mutations were identified when a population of mutagenized animals was screened using the technique of formaldehyde-induced fluorescence for mutants that have unusual patterns of serotonin expression. In *ces-1(n703)* and *ces-2(n732)* mutants, four serotonergic cells were found in the pharynges of the mutant animals instead of just two serotonergic cells that are normally seen in wild-type animals. Analysis of the positions and the lineages of these two extra serotonergic cells indicated that they are sister cells of NSM neurons that fail to die. In addition, the *ces-1(n703gf)* mutation also prevents the death of sister cells of the pharyngeal I2 neurons (21). However, *ces-1* and *ces-2* mutations do not seem to affect the deaths of any other cells, indicating that their activities may be cell-type specific. Two additional *ces-1 gf* alleles and two *cis*-dominant suppressors of *ces-1*(*gf*) mutations, which appear to be loss-of-function alleles of *ces-1*, have been isolated by using Nomarski optics to directly screen for mutations that alter the life or death fate of NSM sister cells (21). In two *ces-1(lf)* mutants, NSM sister cells and I2 sister cells undergo programmed cell death normally as they would in wild-type animals, suggesting that the *ces-1* activity is not required for the deaths of these two types of cells. Only one *ces-2* allele has been identified and is hypomorphic and temperature-sensitive.

The genetic relationship between *ces-1* and *ces-2* has been carefully examined by building double mutants. The *ces-2(lf)* mutation results in the survival of NSM sister cells, suggesting that the normal function of *ces-2* is to cause the death of NSM sister cells. However, in *ces-1(lf) ces-2(lf)* double mutant animals, NSM sister cells undergo programmed cell death normally as they do in wild-type or *ces-1(lf)* animals (21). This observation indicates that the activity of *ces-1* is normally inhibited to allow the death of NSM sister cells and that *ces-2* acts upstream of *ces-1* to suppress the death inhibitory activity of *ces-1* (Figure 4).

The relationship of the *ces* genes with the genes involved in the cell-killing pathway has mainly been inferred from the phenotype of *ces-1*; *egl-1* double mutant. In *ces-1(lf); egl-1(lf)* mutant animals, NSM sister cells survive as they do in the *egl-1(lf)* mutants. This result indicates that *egl-1* most likely acts downstream of *ces-1* to cause cell death and *ces-1* negatively regulates the activity of *egl-1* (23). Thus, *ces-2, ces-1* and *egl-1* function in a negative regulatory chain to regulate the death fate of NSM sister cells. Since the *ces-2(lf)* mutation does not affect the death fate of I2 sister cells, there must be some other gene(s) that acts similarly to regulate the activity of *ces-1* in I2 sister cells.

The above genetic analysis has helped position *ces-1, ces-2* and *egl-1* genes in the cell death pathway. However, elucidation of the mechanism by which this negative regulatory cascade works

to control the death fate of NSM sister cells will require the knowledge of the molecular identities of *ces-1* and *ces-2* genes. *ces-2* has been cloned and found to encode a putative basic leucine-zipper (bZIP) transcription factor (90). This finding suggests that cell death specification in *C. elegans* can be regulated at the level of gene expression.

(2) HSN motor neurons

The regulation of the life vs. death fate of one pair of sex-specific HSN neurons (hermaphrodite-specific-neurons) in *C. elegans* presents another interesting paradigm for studying cell death specification. *C. elegans* has two natural sexes: male and self-reproducing hermaphrodite (1). Two HSN motor neurons control egg laying in hermaphrodites but undergo programmed cell death in males since they are not needed in males (4). Mutations in several genes have been identified that cause the inappropriate death of HSN neurons in hermaphrodites, a male-specific fate of HSN (24, 86, 91). These include gain-of-function mutations in the *her-1* gene (hermaphroditization) and some weak loss-of-function mutations in the *tra-2* gene (sexual transformer), all of which result in partial sexual transformation of some XX hermaphrodites into male-like animals. *her-1* encodes a novel secreted molecule whose activity is high in males to promote male somatic cell fates, whereas the *tra-2* gene encodes a putative transmembrane receptor for the *her-1* protein and whose activity is inhibited by the *her-1* protein in males (92-96). Interestingly, gain-of-function mutations in the *egl-1* gene, which is required for almost all programmed cell deaths in nematodes but not involved in sex determination, also cause ectopic HSN death in hermaphrodite animals (23, 24). Thus *her-1* and *tra-2* may mediate a novel signal transduction pathway that integrates into the cell-death pathway through the *egl-1* gene to control HSN cell death. Several other sex-determining genes, *fem-1, fem-2, fem-3* (feminization) and *tra-1*, have been shown to act downstream of *tra-2* in a negative regulatory cascade to control the somatic sex determination of *C. elegans*, with *tra-1* acting at the last step of this sex-determination pathway (Figure 4; for review see ref 97). Interestingly, *tra-1* encodes a Zn^{2+} finger transcription factor (98). Therefore, the signal transduction pathway that mediates somatic sex determination may control the HSN cell fate by turning on or turning off the expression of key regulatory genes such as *egl-1*. It is intriguing that *egl-1(gf)* mutations do not seem to affect the death of any other cells except that of two HSN neurons (24, 86). It is likely that these *gf* mutations specifically affect the expression or the activity of *egl-1* in HSN neurons. Determination of the molecular lesions of *egl-1*(*gf*) mutations and the expression patterns of the *egl-1* gene in males and hermaphrodites should help understand how *egl-1* is regulated to activate the death program of HSN neurons in a sex-specific manner.

(3) Germline

The cells of *C. elegans* hermaphrodite germline can adopt one of four cell fates: they can undergo mitosis, enter meiosis and differentiate into sperm or oocytes, or they can undergo programmed cell death (for review see ref 99). Germ cell deaths only occur in the adult hermaphrodites and have not been observed in larval stages or in males. Early mitotic and meiotic germ cells in adult hermaphrodites are not completely enclosed by a plasma membrane and instead exist as a large syncytium of asynchronous nuclei in a common cytoplasm (99). Interestingly, direct microscopic observations suggest that nuclei of those dying germ cells rapidly cellularize and separate from the syncitium early in the cell death process, presumably to avoid the diffusion of death-causing factors into the syncitium (9). Over 300 cells in the germline are estimated to die as deduced from the number of unengulfed germ cell corpses observed in engulfment-defective mutant animals (9).

Germ cell differentiation in the adult hermaphrodite corresponds to the position of the germ cell nuclei within the gonad. The gonad of the hermaphrodite animals consists of two U-shaped tubes capped by a somatic distal tip cell (DTC). The stem cell potential of germ cells in close proximity to the DTC is maintained by growth factors secreted by DTC. Germ cells farther away from the DTC enter meiosis and arrest at the pachytene stage. Near the bends of the gonad tubes where germ cell deaths are observed, germ cells receive signals from the Ras/MAPK pathway to exit meiotic arrest and progress to prophase I (99, 100). At this point germ cells that do not die increase in size to form oocytes as they migrate towards the uterus.

Phenotypic analysis of mutations that affect sexual identity or germ cell differentiation has shown that germ cell deaths are associated with oogenesis and not spermatogenesis or mitosis (9). For example, *tra-1* loss-of-function mutant XX animals, which are incapable of producing oocytes, have no observable germline programmed cell death. Furthermore, no programmed cell death is seen in *mog-1* (masculinization of germline) loss-of-function mutants, in which the germline of XX hermaphrodites is masculinized such that only sperm are produced. Interestingly, entry in meiosis appears to be a requirement for germ cell death. For example, in *gld-1* (germline differentiation abnormal) mutant hermaphrodites, in which meiotic differentiation is blocked, no germ cell deaths are observed. These results suggest that only meiotic oogenic germ cells undergo programmed cell death (9).

As mentioned above, activation of the Ras/MAPK signaling cascade is required for exit of germ cells from meiotic arrest (99, 100). Loss-of-function mutations in several *genes* [*let-60(ras), lin-45(raf), mek-2*(MAPK kinase) and *mpk-1(*MAP kinase*);* 101] in the *ras* signaling pathway block the ability of germ cells to complete meiosis. Additionally, the same loss-of-function mutations almost completely abolish germ cell deaths (9). Thus, exit from meiotic arrest is a prerequisite for germ cells to undergo programmed cell death.

Programmed deaths of somatic and germ cells are morphologically similar, except that germ cell corpses usually are larger in size than somatic cell corpses. Both types of cell deaths are characterized by condensation of cytoplasm and nuclei, aggregation of chromatin, and rapid recognition and phagocytosis of cell corpses by surrounding cells (2, 9, 13). The kinetics of both cell deaths also appears to be similar. From the initiation of the germ cell death to the engulfment of the germ cell corpse by gonadal sheath cells that surround the germline, the whole process is completed in less than one hour, a time frame comparable to that of somatic cell deaths (2, 9).

Genetic analysis suggests that somatic and germ cell deaths share components of the cell-killing machinery. For example, the cell killing activities of *ced-3* and *ced-4* not only are required for the execution of somatic cell deaths but also required for the execution of germ cell deaths (9). Loss-of-function mutations in *ced-3* or *ced-4* abolish all germ cell deaths. In addition, *ced-9* is required to protect germ cells from death: many more germ cells undergo programmed cell death in *ced-9(lf)* mutants. However, this general cell death program mediated by *ced-3, ced-4* and *ced-9* appears to be regulated differently in somatic and germ cells. For example, the *egl-1* gene, which is required for the activation of almost all somatic cell deaths, is not required for the activation of germ cell deaths, since in the *egl-1*(*lf*) mutants germ cell deaths are not blocked. In addition, *ced-9* (*n1950, gf*) mutation that prevents almost all somatic cell deaths has little effect on germ cell deaths (9). These observations suggest that *ced-9* may interact with a different cell death initiator to regulate the activation of cell death in germline.

One interesting question regarding the germ cell death is why germ cells undergo programmed cell death. One possibility is that it is used as a mechanism to eliminate unfit germ cells. This seems unlikely since in *ced-3* or *ced-4* mutant animals there is no increase in the number of defective oocytes or embryonic lethality (9). A more favored explanation is that many germ cells in *C. elegans* are generated in extra to serve as nurse cells to synthesize cytoplasmic components for mature oocytes. Once they fulfill this function, they are eliminated by cell death.

(4) Cell deaths in other specific cell types

Although much is known about the killing step of programmed cell death in *C.elegans*, little is known about how cells make the life vs. death decisions. The cases discussed above describe only a fraction of the programmed cell deaths that occur in *C.elegans*. The regulation of the life vs. death decisions of many other cells in *C.elegans* remains an important and challenging question.

2.4 Genes involved in the engulfment of cell corpses

Once cells undergo programmed cell death, their corpses are swiftly engulfed and degraded, usually within an hour (2, 14). Unlike *Drosophila malanogaster* and mammals, *C. elegans* does not have professional phagocytes that recognize and phagocytose dying cells. Instead, cell corpses

are engulfed by their neighboring cells that directly contact them. Cell corpses generated during embryogenesis are mostly engulfed by their siblings (4), while postembryonic cell corpses are removed by the hypodermis, a large hypodermal syncytium that envelops most of the animal. The engulfment-inducing signal in dying cells appears to be expressed at a very early stage of the death process: pseudopodia have been observed to extend from an engulfing cell around the dying cell even before the cell division generating the dying cell has completed (13).

What is the signal that marks the dying cell? How does this signal trigger phagocytosis? What is the mechanism of phagocytosis? Genetic and molecular studies of the cell-corpse engulfment process in *C. elegans* have provided some valuable information towards answering some of the questions.

(1) Genetic analysis of genes involved in the engulfment process

At least six genes, *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7* and *ced-10* have been identified to be involved in cell-corpse engulfment (14, 20). Mutations in any of these genes block the engulfment of many dying cells and result in the phenotype of persistent cell corpses (Figure 3). Since the cells destined to die still die in the engulfment-defective mutants, the engulfment process *per se* does not cause cell death. Instead, it functions to remove corpses after cell death occurs. Genetic analysis suggests that these six genes fall into two groups: *ced-1*, *ced-6* and *ced-7* in one group and *ced-2*, *ced-5* and *ced-10* in the other (20). Single mutants or double mutants within the same group show certain degree of engulfment defects, while double mutants between two groups show more severe engulfment defects. One model consistent with these genetic data is that these two groups of genes are involved in two distinct but partially redundant pathways that lead to the recognition and subsequent phagocytosis of cell corpses by engulfing cells (20). It is possible that dying cells exhibit two different engulfment-inducing signals recognized by distinct molecules on engulfing cells and that only when both signaling systems are engaged can phagocytosis be efficient. All the engulfment genes except *ced-1* show maternal rescue for the engulfment of embryonic deaths (20), suggesting that the maternal contribution of these engulfment gene products in either embryos or germline is sufficient for engulfment to take place. However, postembryonic somatic cell deaths do not show maternal rescue.

Mutations in *ced-2, ced-5,* and *ced-10* also cause defects in distal tip cell (DTC) migration (27, 102). DTCs are located at the tip of each gonadal arm and guide the extension of gonadal arms during larval development (Figure 6A). In *ced-2, ced-5* and *ced-10* mutants, DTCs frequently make extra turns or stop migration prematurely, resulting in abnormally shaped gonad in adult animals (Figure 6B).

One other feature shared by *ced-2, ced-5* and *ced-10* mutations is that they can suppress abnormal cell deaths caused by semi-dominant (*sd*) mutations in two genes *lin-24* and *lin-33*

(lineage abnormal). In *lin-24(sd)* and *lin-33(sd)* mutants, P1.p-P12.p (collectively designated Pn.p) cells, which lie in the ventral midline and produce hypodermal and vulval cells, display abnormal morphologies and eventually degenerate (103-105). The death induced by *lin-24(sd)* and *lin-33(sd)* mutations does not seem to occur via normal programmed cell death pathway, for three reasons. First, these deaths do not show the typical morphological changes of programmed cell death as observed by Nomarski optics or in the EM ultrastructural studies (105). Second, mutations in *ced-3* and *ced-4* do not block these abnormal deaths (18). Third, these deaths appear to require the activities of three engulfment genes *ced-2, ced-5* and *ced-10*, since mutations in any of the three genes can suppress abnormal deaths in *lin-24(sd*) and *lin-33*(*sd*) mutants (105). It is possible that *lin-24(sd*) and *lin-33(sd*) mutations cause the Pn.p cells to be recognized and engulfed by their neighboring cells as dying cells, and that the activities of *ced-2, ced-5* and *ced-10*, but not those of *ced-1, ced-6* and *ced-7* are required for phagocytosis of these dying cells. If this is true, *ced-2*, *ced-5* and ced10 genes might be involved in eliminating cells undergoing programmed cell death as well as abnormal cell deaths (27). Alternatively, the products of *ced-2, ced-5* and *ced-10* may directly contribute to the abnormal death of Pn.p cells in *lin-24(sd*) and *lin-33(sd)* mutants which has nothing to do with the engulfment function of these three genes. The molecular identities of the *lin-24* and *lin-33* genes and the toxic nature of the LIN-24(*sd*) and LIN-33(*sd*) proteins have yet to be determined.

These six engulfment genes identified so far may not represent all the genes involved in cell corpse engulfment. It has been reported that animals homozygous for some genomic deficiencies exhibit the phenotypes of increased numbers of unengulfed cell corpses and embryonic lethality (106), suggesting that other unidentified genes may also function in cell corpse engulfment.

(2) Molecular analysis of genes involved in the engulfment process

Of the six engulfment genes identified so far, three (*ced-5*, *ced-6* and *ced-7*) have been cloned. Below we will describe the molecular characterization of these genes and the potential roles they may play during cell-corpse engulfment.

(a) *ced-5*

The engulfment defect of the *ced-5* mutant can be rescued by using heat-shock promoters to induce the expression of a wild-type *ced-5* transgene (102). The rescue can be achieved even when cell corpses are long dead for hours or even days in a *ced-5* mutant, suggesting that the engulfment-inducing signal(s) is stable and can stay functional for a long time with the cell corspes. The transcription or/and translation machinery of such late cell corpses appears to be inactive since expression of green fluorescent protein (GFP) can not be induced in these cell corpses by the heat-shock treatment (102). Therefore, the eventual engulfment of these late corpses by induced

expression of *ced-5* is likely effected by *ced-5* expression in engulfing cells rather than in cell corpses. These observations suggest that *ced-5* functions in engulfing cells but not in dying cells.

How does *ced-5* function in engulfing cells to mediate cell-corpse engulfment? The clue comes from the molecular identification of the *ced-5* gene. *ced-5* encodes a protein similar to human DOCK180 (107), *Drosophila* Myoblast City (MBC; 109), the predicted protein sequence from a human cDNA clone KIAA0209 (108), the yeast open reading frame L9576.7 (GeneBank accession number 664878), and a mouse expressed sequence tag (GeneBank accession number AA110899). CED-5 is most similar to DOCK180; these two proteins share 26% identity throughout their entire lengths. Both DOCK180 and MBC have been implicated in mediating the extension of cell surfaces (107, 109, 110). Interestingly, CRK, a DOCK180 interacting adaptor protein is involved in integrin-mediated signaling (111), and vitronectin, a member of the integrin superfamily, has been implicated in cell-corpse engulfment in mammals (112).

Expression of human DOCK180 in *C. elegans* can rescue the cell-migration defect of a *ced-5* mutant, suggesting that DOCK180 and CED-5 are, at least in this aspect, functionally interchangeable (102). However, expression of DOCK180 in *C. elegans* failed to rescue the *ced-5* engulfment defect. It is possible that DOCK180 possesses the function required for DTC migration but not that for cell-corpse engulfment. It is also possible that DOCK180 does have a role in cell-corpse engulfment but the sequence similarity with CED-5 is not good enough for it to interact efficiently with other components in the CED-5 pathway. Elucidation of the physiological role of DOCK180 in mammals will help distinguish these two possibilities.

The molecular characterization of the *ced-5* gene and the phenotypic analysis of *ced-5* mutants altogether suggest that *ced-5* functions in mediating the extension of cell surfaces as engulfing cells phagocytose cell corpses and as migrating DTCs move along body wall muscles. *ced-5* mutants appear to be normal in the migration of other cells (e.g., the migrations of P1-P12 precursor cells and the HSN neurons) as well as in the axonal outgrowth of neurons and in cell fusion that is involved in the development of the hypodermal syncytium (20, 102). Thus, *ced-5* is likely to function in a specific type of membrane extension that is common to both cell-corpse engulfment and DTC migration.

Like *ced-5*, the *ced-2* and *ced-10* genes are also important for cell-corpse engulfment as well as for normal DTC migration. Therefore, it is possible that these two genes also function in membrane extensions required for these two processes.

(2) *ced-7*

CED-7 protein has sequence similarity to ABC (ATP-binding cassette) transporters (113). ABC transporters have been found to mediate transport of diverse substrates, including irons, sugars, vitamines, phospholipids, peptides, and proteins (114, 115). The mechanism by which each

ABC transporter achieves its substrate specificity is not clear. One characteristic feature of ABC transporters is the unidirectionality of substrate transport (114, 115). The transport process appears to be export rather than import in almost all eukaryotic ABC transporters with identified substrates, except CFTR, which acts as a chloride channel (114). Whether *ced-7* can function as a transporter, and if so, what are its physiological substrates, remain to be determined.

CED-7 is most similar to the ABC1 subfamily proteins, which include the mouse ABC1 and ABC2 (116), the human ABC-C (ABC3) and ABCR (117-119) and the bovine rim proteins (120). CED-7 is most similar to the ABC-C protein. These two proteins share 25% identities along their entire lengths. Among the ABC transporters that have been identified thus far, the ABC1 transporter, which is 20% identical to CED-7, has been implicated in the engulfment of mammalian apoptotic cells (102, 121). The mechanism by which ABC1 mediates engulfment of apoptotic cells is still not clear.

The CED-7 protein is widely expressed during embryogenesis as studied using anti-CED-7 antibodies (102). This broad expression pattern suggests that the activity of CED-7 should be tightly regulated so that *ced-7*-mediated engulfment can specifically target dying rather than viable cells. Mosaic analysis revealed that CED-7 functions in both dying cells and engulfing cells during the engulfment process (102). This finding and the observation that CED-7 is localized to the plasma membrane suggest that CED-7 activity may be important for the interaction between the cell surfaces of the dying and engulfing cells. Such interaction may be important for two aspects of engulfment: 1) the recognition of a dying cell by an engulfing cell, and 2) the adhesion between two cell surfaces as an engulfing cell extends pseudopodia around a dying cell during phagocytosis. If *ced-7* indeed functions as a transporter, it is unlikely that *ced-7* functions in the recognition process unless *ced-7* transports different molecules in dying cells and living cells. It has been reported that some ABC transporters can transport hydrophobic substrates (114). One simple and more favored model is that CED-7 functions to transport a substrate that mediates homotypic adhesion between the dying and engulfing cells. Identification of CED-7 substrate(s) should help understand the mechanism underlying the *ced-7*-mediated engulfment process.

(3) *ced-6*

ced-6 might act downstream of or in parallel to *ced-1* and *ced-7* since overexpression of *ced-6* can partially rescue the engulfment defect in *ced-1* and *ced-7* mutants but not that in *ced-2* and *ced-5* mutants (122). The CED-6 protein contains two potential functional motifs: a phosphotyrosine-binding (PTB) domain and a proline/serine-rich region that may interact with the SH3 (Src homology 3) domain of signaling proteins (122-124). PTB domains can recognize a phosphorylated tyrosine residue within a NPXY consensus sequence (X: any amino acid) (124, 125). Intriguingly, the CED-7 protein contains a NPLY sequence in one of its predicted

cytoplasmic domains. Whether CED-7 can be phosphorylated on this tyrosine residue and hence recruit CED-6 to the membrane remains to be determined. Genetic mosaic analysis demonstrates that *ced-6* acts within engulfing cells (122). This finding and the presence of a PTB domain and potential SH3-binding sites in CED-6 suggest that CED-6 may serve as an adaptor protein to directly or indirectly transduce a signal from a receptor to effectors or cytoskeletal proteins to initiate the rearrangement of cytoskeleton organization and the phagocytosis process.

Although genetic screens for engulfment mutants have not been saturated (20) and the molecular identities of three other engulfment genes (*ced-1, ced-2* and *ced-10*) have yet to be determined, the molecular characterizations of *ced-5, ced-6* and *ced-7* genes should open up more avenues for studying the mechanisms by which cell corpses are engulfed.

2.5. Genes involved in degradation of cell corpses

Degradation of cell corpses appears to require the involvement of engulfing cells in *C. elegans*, since cell corpses persist in engulfment-defective mutants. In *ced-1* and *ced-2* mutants, Feulgen-reactive material (Feulgen stains DNA) is visible in persistent cell corpses, indicating that DNA degradation does not proceed to completion (14). Thus, complete degradation of chromosomal DNA of dead cells requires the activities of the *ced-1* and *ced-2* genes or an activity provided by engulfing cells.

The *C. elegans* gene, *nuc-1* (nuclease), appears to be required for DNA degradation of dead cells but not for the execution of cell death or for the engulfment of cell corpses (17). In *nuc-1* mutants, both cell death and engulfment occur, but DNA from the engulfed dead cells is not degraded and persists as a compact mass of Fuelgen-reactive material (14, 17). The *nuc-1* gene is also involved in digesting the DNA of bacteria on which the animals feed, since bacterial DNA can be detected in the intestinal lumena of *nuc-1* mutants, but not in those of wild-type animals (17). Biochemical studies of *nuc-1* mutants indicated that an endonuclease activity that is present in wild-type animals is reduced to 1% in *nuc-1* animals (126). *nuc-1* may thus encode an endonulease or a protein that controls the activity of the endonulcease.

Although nucleosomal DNA ladders, a hallmark of apoptosis, have not been shown to occur in *C. elegans*, DNA degradation by endonuclease(s) which generate 5'-phosphate and 3'-hydroxyl ends has been detected by TUNEL staining *in situ* (Y.C. Wu, G. M. Stanfield, and H. R. Horvitz, personal communication), a technique that has been used widely to identify dying cells (127). Since the time and the position of each cell doomed to die is known in *C. elegans*, the studies of TUNEL-staining patterns should help understand the kinetics of DNA degradation *in vivo.*

3. Conclusion and future perspectives

3.1 Conservation of the cell-death pathway between nematodes and mammals

Programmed cell death is a widespread phenomenon in the animal kingdom and an essential cellular process in animal development and homeostasis (25, 128). Thus it has been speculated that such an important process may be evolutionarily conserved. In the last two sections we described genetic, cellular, and molecular studies of programmed cell death in *C. elegans*. These studies have led to three major conclusions: 1) Programmed cell death in *C. elegans* undergoes morphological changes very similar to those of cell deaths in other species including human. 2) Many components of the *C. elegans* cell death pathway, especially those involved in the cell-killing process, have sequence homologs that play similar roles in mediating apoptosis in other species. This conclusion is underscored by the sequence and functional similarities between CED-3 and caspases, CED-4 and Apaf-1, CED-9 and Bcl-2 family proteins, and EGL-1 and BH3-only Bcl-2 family proteins. Furthermore, expression of some of these proteins in foreign species could exert similar effects on cell death as they do in their native species. For example, expression of the human *bcl-2* gene an efficiently inhibit cell death in *C. elegans* and even substitute for the function of *ced-9* in *C. elegans* (54, 71, 77). On the other hand, expression of CED-3 can efficiently cause apoptosis in mammalian cells or insect cells (61,129). 3) The basic mechanisms by which cell death is regulated and executed are likely to be conserved. For example, baculovirus p35 protein can inhibit cell death in diverse species by inhibiting the activities of CED-3 and death caspases (52, 53). And the activation of CED-3 and caspase proenzymes is likely to be induced by conserved oligomerization mechanisms such as receptor oligomerization or adaptor protein (Apaf-1 and CED-4) oligomerization (41-45, 67). All these studies firmly establish that the cell death pathway is conserved between nematodes and mammals. Given the simplicity of the cell death machinery in *C. elegans* (e.g., only four caspases including CED-3 identified so far in *C. elegans* in comparison with sixteen found in mammals; 130) and the powerful genetic and molecular biology tools available, the study of programmed cell death in *C. elegans* is likely to provide important insights into the mechanisms of programmed cell death in general.

3.2 Key issues of *C. elegans* programmed cell death

In the last few years, molecular and biochemical studies of *C. elegans* cell-death genes identified by genetic analyses have greatly improved our understanding of how cell death is initiated and executed. However, many important questions remain to be answered regarding three aspects of programmed cell death in nematodes: 1) the decision making process of which cell should die, 2) the activation of the cell death program, and 3) the execution of cell death process. In the following, we will discuss what we think are the most important issues that need to be addressed in the future.

(1) Genes or pathways that determine which cells should die in *C. elegans*

During the development of adult hermaphrodites, 131 somatic cells invariantly die by programmed cell death (2, 4). With the exception of a few cell types (sister cells of NSM neurons and HSN neurons), the mechanisms that specify the deaths of most of the 131 cells are largely unknown. The controls of the cell-death fate of NSM sister cells and the life vs. death fate of HSN neurons in different sexes have provided two interesting examples on how cell death specification in *C. elegans* may be regulated as well as exciting future prospects of studying cell death regulation. Intriguingly, cell-death specifications in these two cell types both involve negative regulatory cascades. Such regulatory schemes are also seen in the central cell-killing pathway (Figure 4). It will be interesting to see whether cell-death specifications in other cell types involve similar strategies.

Like *ces-1* and *ces-2* mutations, mutations in other cell-death-specification genes may specifically affect the life vs. death fates of a small number of cells. Such mutations may fail to cause any visible phenotype that can be scored in a conventional genetic screen using a dissecting microscope. This may explain why only a few *ces* genes have been identified so far. Therefore, direct screens using Nomarski optics to look for mutant animals that have abnormal survival or death of a particular cell(s) may be necessary. However, such a screening process is rather tedious and time-consuming. To increase the efficiency of identifying new *ces* genes, it will be important to develop novel methods that will allow the screen to be done using a dissecting microscope, such as using GFP (green fluorescence protein; 131) to mark a specific cell lineage or cell type or identifying a visible phenotype associated with the ectopic death of a particular cell(s).

(2) Regulation of the *egl-1* death-activating activity.

As a gene that sits at the beginning of the central cell-killing pathway, *egl-1* may play a crucial role in receiving and integrating death signals into the cell death pathway. How does *egl-1* play such a role? It is conceivable that the expression of the *egl-1* gene can be turned on or upregulated in response to the death cues. It is also possible that the activation of *egl-1* can be achieved at the post-translational level by modifications of EGL-1 such as phosphorylation or cleavage by a protease. Recent studies have suggested that the death-inducing activities of Bid and Bim, both of which are mammalian BH3-only proteins like EGL-1, are activated through proteolytic cleavage of Bid by caspase-8 or dissociation of Bim from the dynein motor complex (132-135). Examination of the temporal and spacial expression patterns of *egl-1* and biochemical analysis of the EGL-1 protein will help address these questions.

(3) The biochemical function of CED-9

Like the Bcl-2 family of anti-apoptotic proteins, the biochemical function of CED-9 and the mechanism by which CED-9 acts to inhibit cell death remain a mystery. Does CED-9 simply serve

as a sequestering protein to prevent CED-4 from activating CED-3 as some have suggested (59, 64) or does it employ a more complicated mechanism to inhibit CED-4 activity? Why does *ced-9(n1950, gf)* mutation result in more potent protective activity of CED-9 and why does this mutation inhibit somatic cell deaths but not germline cell deaths? Is CED-9 localized to *C. elegans* mitochondria and/or other organelles as Bcl-2 is? Does CED-9 have the pore-forming ability on membranes like Bcl-XL and Bcl-2? A related and more fundamental question is whether mitochondria is involved in regulating *C. elegans* programmed cell death. If so, is cytochrome c or an equivalent molecule from mitochondria involved? So far, there is no direct evidence that is suggestive of the involvement of mitochondria in *C. elegans* cell death. Studies of subcellular localization of CED-9 and the nature of its interactions with other cell death proteins such as EGL-1 and CED-4 may help answer some of these puzzles.

(4) The biochemical function of CED-4

CED-4 and the mammalian caspase activator Apaf-1 have sequence similarity over a stretch of 320 amino acids that contains two adenine triphosphate binding motifs (46, 55). Apaf-1, in addition, has twelve or thirteen WD-40 repeats at its carboxyl terminus that are not found in CED-4 (67). *In vitro*, dATP (or ATP) and cytochrome c are necessary and sufficient for Apaf-1 to activate procaspase-9 (66, 67). However, cytochrome c and dATP become dispensable if the WD-40 repeat region is deleted from Apaf-1, suggesting that the WD-40 repeat region acts as a negative regulatory domain for Apaf-1 (41, 136). It is not clear whether CED-4 alone is sufficient to activate CED-3 or whether it needs other co-factor(s) to do it. Since CED-4 does not contain the WD-40 repeat region, cytochrome c may not be involved in the activation of CED-3. But this does not rule out the possibility that another factor (s) is needed. One relevant observation is that *ced-4* kills very poorly when ectopically expressed in those cells that normally live in *C. elegans* (40). This poor killing by *ced-4* may be due to either the presence of a death inhibitory factor like CED-9 or the absence of a CED-4 co-factor in these normally living cells. The fact that the cell killing caused by overexpression of *ced-4* can be enhanced to a certain level in the absence of the endogenous CED-9 product lends support for the first scenario but still leaves the second possibility open. The second unanswered question regarding CED-4 is whether it hydrolyzes dATP (or ATP) and whether the hydrolysis of dATP (or ATP) is required for its activity. dATP or ATP hydrolysis is required for the activation of pro-caspase9 by the full-length Apaf-1, since nonhydrolyzable analogue of ATP blocks this activation process (66, 67). Furthermore, mutations at the highly conserved residues of the Walker's A motif of these two proteins abolish the activity of CED-4 and Apaf-1 (61, 62, 67, 136), indicating that the hydrolysis of dATP or ATP is also likely to be important for CED-4 function. Establishment of an *in vitro* assay for CED-4 activity, both its putative ATPase activity

and its CED-3-activating activity, will be important for understanding how CED-4 acts to cause cell death in *C. elegans*.

(5) The mechanisms that activate the CED-3 death protease

The activation of the CED-3 proenzyme marks the point of no return for programmed cell death. Thus this process has to be tightly controlled so that CED-3 is activated only in the cells that are doomed to die. As we have discussed above, CED-3 is expressed probably in many cells as a 56 kD protease precursor and then proteolytically activated to generate an active protease that is composed of p17 and p15 (or p13) subunits in those cells fated to die. The observations that the CED-3 precursor can be cleaved exactly at the same sites by active CED-3 protease *in vitro* to generate p17/15 or p17/p13 protease subunits and that forced dimerization of CED-3 precursors results in the activation of CED-3 *in vitro* suggest that CED-3 precursor molecules may be able to cleave and activate one another if brought into close proximity by some mechanisms, similar to what have been proposed for the activation of procaspases in mammals (35, 45, 128). What mechanisms could bring CED-3 into close proximity? Increased concentration of CED-3 in cells could enhance the chance that two CED-3 molecules contact each other. This may explain why CED-3 can be activated if overexpressed in bacteria or in *C. elegans*. Oligomerization of CED-4 may also bring CED-3 to proximity and may be the main mechanism used to activate CED-3 in *C. elegans*, especially in those cells fated to die. Finally, CED-3 may also be cleaved and activated by other caspases. Three additional *C. elegans* caspases have been identified, one of which can process CED-3 *in vitro* (130). However, whether these three *C. elegans* caspases have any role in programmed cell death or in CED-3 activation remains to be determined. The study of when and where *ced-3* is expressed, the level of *ced-3* expression and its subcellular localization in dying cell and living cells, and the expression patterns of *ced-3* in different cell death mutants will be important for understanding how CED-3 is activated.

(6) The substrates or targets of the CED-3 death protease

After CED-3 is activated, it will cleave its substrates or targets to initiate morphological and cytological changes in dying cells that eventually lead to the engulfment and degradation of cell corpses by neighboring cells. What are these substrates? It is conceivable that some of these substrates need to be cleaved and activated to initiate certain aspects of the cell death execution process. For example, PAK-2 kinase (p21 activated kinase 2) activity is activated by caspase cleavage to mediate apoptotic body formation (137). By contrast, some cell death inhibitors need to be cleaved and inactivated so that cell death can proceed swiftly without resistance, much like the cleavage and inactivation of ICAD (inhibitor of CAD) by caspase-3 to allow fragmentation of chromosomal DNA by CAD (caspase-activated deoxyribonuclease; (138). In addition, some

substrates are cleaved to ensure that those machinery that maintain cellular homeostasis (e.g. translation or transcription), which become unnecessary in dying cells, are shut down to further facilitate the death of cells (139). Identification of the death-protease substrates apparently is important for understanding the mechanisms that control the execution of cell death. So far, many proteins have been implicated as substrates of the death proteases (139); however, few of them have been shown to be involved in cell death *in vivo*. In *C. elegans*, CED-9 and the CED-3 precursor itself have been shown to be substrates of the CED-3 protease (53, 54). The CED-3 cleavage sites in CED-9 are important for the full death protective activity of CED-9 in *C. elegans* . Genetically substrates of CED-3 should act downstream of the *ced-3* gene in the genetic pathway of programmed cell death. Most of the genes identified so far that act downstream of *ced-3* are components that function at the late stage of programmed cell death, when cell deaths have already occurred (Figure 4; 25). These include genes that act in the cell-corpse-engulfment process and the *nucl-1* gene that is involved in the degradation of DNA from death cells (25). No gene appears to act immediately downstream of *ced-3*. Given that CED-3 causes cell death by cleaving multiple substrates which may lead to simultaneous activation of many facets of the cell death execution process, elimination of one of the CED-3 substrates by genetic mutations may not result in obvious cell death defects that could be detected in previous genetic screens. Instead, these mutants may partially suppress cell death or delay cell death. Using this criteria for genetic screens, several new genes have been identified that may act immediately downstream of *ced-3* to execute cell death (D. Ledwich and D. Xue, unpublished results). Genetic screens of this kind should help identify the molecular components and the genetic pathways that mediate the execution of cell death by the CED-3 protease.

(7) The recognition and the engulfment of dying cells

Although the molecular cloning of the *ced-5*, *ced-6*, and *ced-7* genes has shed some light on what molecules might be involved in the cell-corpse-engulfment process. Several key questions remain unanswered: (1) What are the engulfment-inducing signals and how are they generated by dying cells? (2) What are the receptors that recognize the engulfment-inducing signals? (3) How are the engulfment-inducing signals transduced from receptors to the phagocytosis machinery of engulfing cells to initiate the engulfment of dying cells?

Since CED-3 death protease executes cell death by cleaving critical substrates, it is possible that engulfment-inducing signals are generated by CED-3 protease cleavage. However, it is also possible that the signals could be generated by a mechanism that is not dependent on direct cleavage by CED-3, but rather, products generated during the subsequent cell disassembly process. For example, the exposure of phosphotidylserine on the outer leaflet of plasma membrane due to the

loss of plasma membrane asymmetry during apoptosis has been shown to be important for recognition of apoptotic cells by macrophages *in vitro* (140, 141).

Molecular and genetic analyses of the *ced-5* and *ced-6* genes suggest that they may act in engulfing cells to transduce the signals that trigger the phagocytosis of dying cells. How are these two proteins linked to the receptors that recognize dying cells and to the cellular machinery that executes the phagocytosis process? Molecular characterization of the remaining engulfment genes (*ced-1*, *ced-2* and *ced-10*) should provide additional information towards answering these questions.

(8) Others

In addition to the above major issues listed, there are a few other important questions that also need to be addressed. For example, what is the gene that activates the cell death program in germ cells as *egl-1* does in somatic cells? How are the levels of CED-4S and CED-4L differentially controlled in the living cells and dying cells? What is the molecular basis for the killing activity of *ced-9* in cells that normally die? Answers to these questions undoubtedly will significantly improve our understanding of programmed cell death.

3.3 Concluding remarks

In the last few years, we have witnessed remarkable progresses towards the understanding of basic mechanisms that guide the activation and execution of programmed cell death. Genetic studies in *C. elegans* have led to the identification of a genetic pathway for programmed cell death and have provided a genetic framework for studying the regulation and execution of this important and fascinating process. Molecular and biochemical analyses of key components of this cell death pathway demonstrate that the cell death pathway is highly conserved and have provided invaluable insights into the mechanisms of programmed cell death in general. With the completion of genome sequence of *C. elegans* and the emergence of powerful techniques such as dsRNAi (double stranded RNA interference; 142) that allow study of the functions of virtually any *C. elegans* gene, the genetic and molecular studies of programmed cell death in *C. elegans* will continue to contribute in a major way to the deciphering of mechanisms of programmed cell death.

Figure legends

Figure 1. Morphological changes of a dying cell during the cell death process as viewed with Nomarski optics. A dying cell, P11.aap from a L1 hermaphrodite, is indicated by an arrow. The cell shows maximum refractility at approximately 28 minutes after its birth (upper right panel). The scale bar represents 20 μm. (Reprinted, with permission from Sulston and Horvitz, 1977).

Figure 2. Asymmetric distribution of programmed cell deaths among the lineages of the *C. elegans* hermaphrodite. Early divisions represented by horizontal lines produce six founder cells-AB, MS, E, C, D and P4. The number of cells generated and the number of cell deaths observed from each cell lineage are indicated. The AB lineage generates hypodermis, neurons and muscle. The MS lineage generates muscle, glands and neurons. The C lineage generates hypodermis, neurons and muscle. The D and E lineages produce only gut and muscle, respectively. The P4 lineage gives rise to variable number of cells and cell deaths in the germline.

Figure 3. An engulfment-defective mutant exhibits persistent cell corpses. (A) Nomarski micrograph of a wild-type four-fold embryo (~700 min) that lacks cell corpses. Only the anterior two thirds of the embryo is in focus. (B) Nomarski micrograph of a four-fold embryo of a *ced-1(e1735)* mutant with many persistent cell corpses (indicated by arrowheads). The scale bar represents 10 μm.

Figure 4. The genetic pathway of programmed cell death in *C. elegans*. Four sequential steps of programmed cell death are indicated. In the cell-death-specification step, genes that are involved in regulating the death fates of three specific cell types (sister cells of NSM neurons, HSN neurons and germline) are shown. In germline, activation of the *ras*/MAP kinase pathway promotes the exit of germ cells from the meiotic arrest. Some of these germ cells proceed to undergo programmed cell death. There are two partially redundant pathways (*ced-1, 6, 7* and *ced-2, 5, 10*, respectively) that mediate the engulfment of cell corpses. The position of the *ced-8* gene in the cell death pathway is not clear.

Figure 5. Molecular model for the activation of CED-3 during programmed cell death. (A) In cells that normally live, CED-3, CED-4 and CED-9 form a ternary protein complex associated with mitochondria, in which CED-3 remains inactive. CED-3 may also directly contact CED-9 given that CED-9 has two CED-3 cleavage sites. (B) In cells that are fated to die, EGL-1 displaces CED-4/CED-3 complex from CED-9, leading to the oligomerization of CED-4/CED-3 complex. (C) Oligomerized CED-4/CED-3 complex facilitates the processing and activation of CED-3.

Figure 6. Schematic drawings of the DTC locations and gonadal shapes observed in wild-type and *ced-5* adult hermaphrodites. (A) DTC locations and gonadal shape observed in the wild-type hermaphrodite. (B) An abnormally-shaped gonad observed in a *ced-5* mutant, resulting from an extra turn made by the anterior DTC and the premature termination of the posterior DTC during the migration process. The gonad is shaded in grey, and the DTCs are indicated by black circles.

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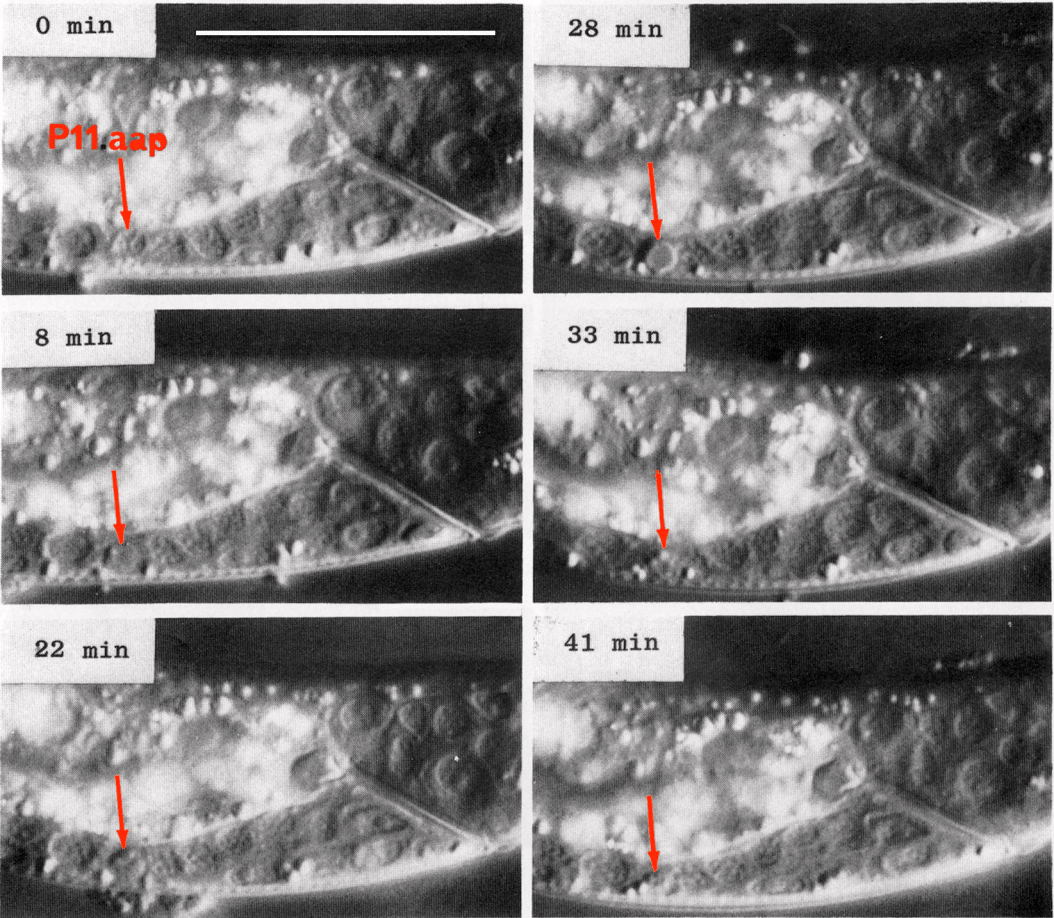
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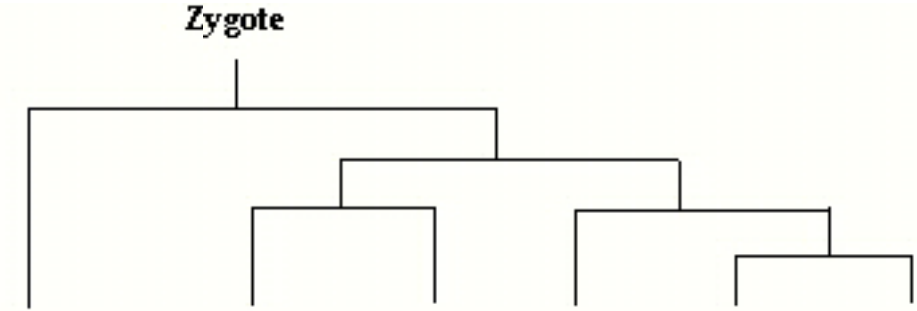

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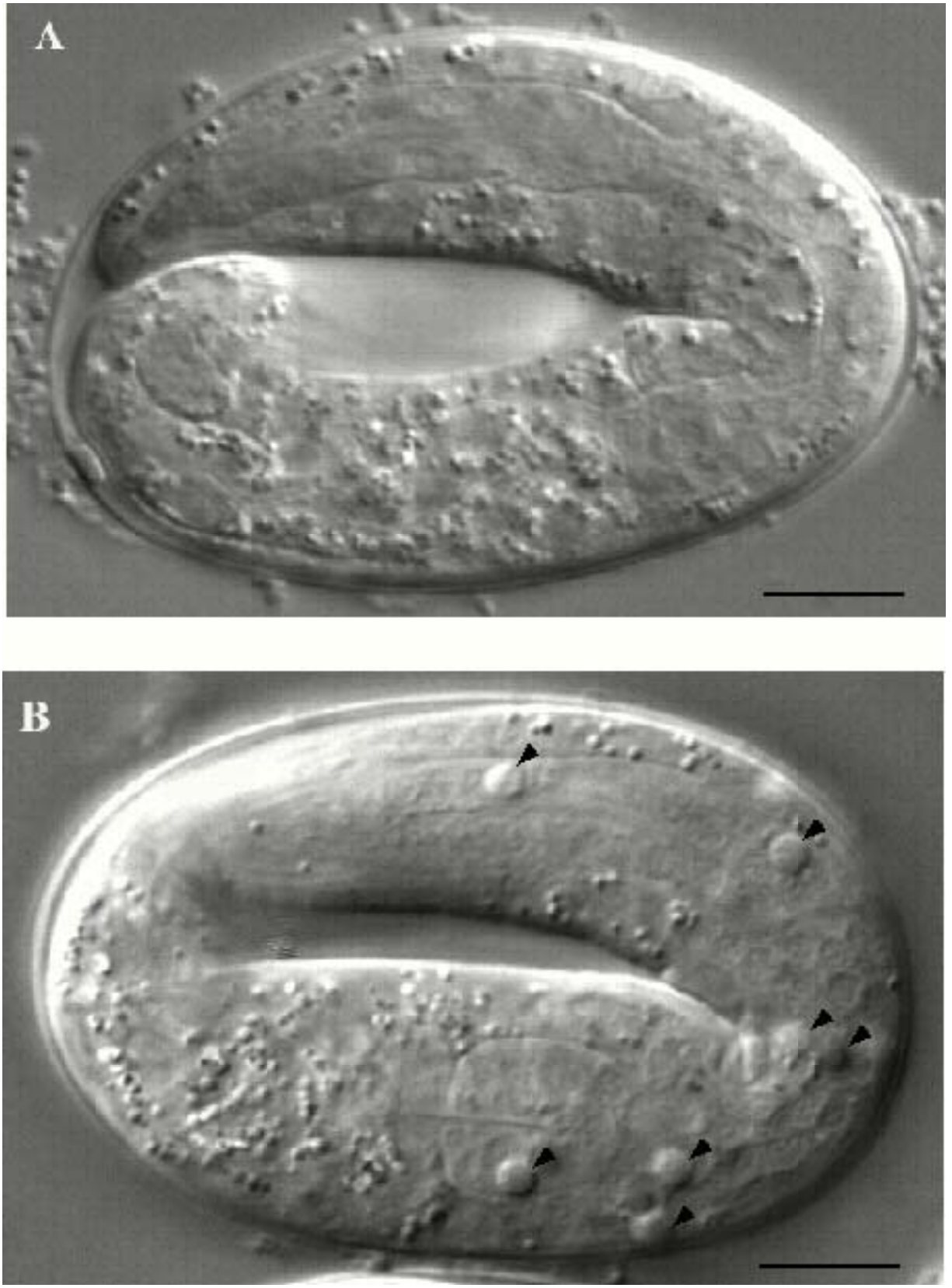
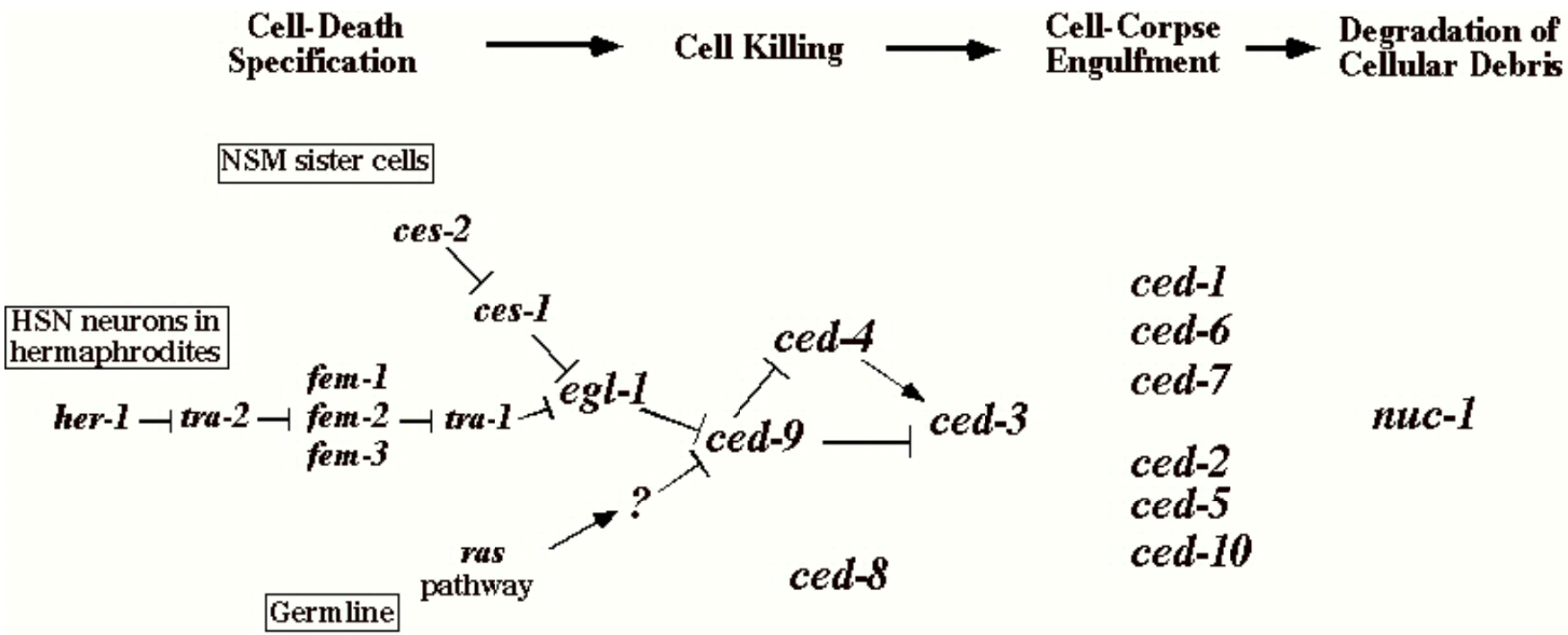
No. programmed cell deaths	116	14	0	1	0	variable


A
B


Cell-Death Specification
Cell Killing
Cell-Corpse Engulfment
Degradation of Cellular Debris
NSM sister cells
ces-2
ces-1
HSN neurons in hermaphrodites
her-1
tra-2
fem-1
fem-2
fem-3
tra-1
egl-1
ced-4
ced-9
ced-3
?
ras pathway
Germline
ced-8
ced-1
ced-6
ced-7
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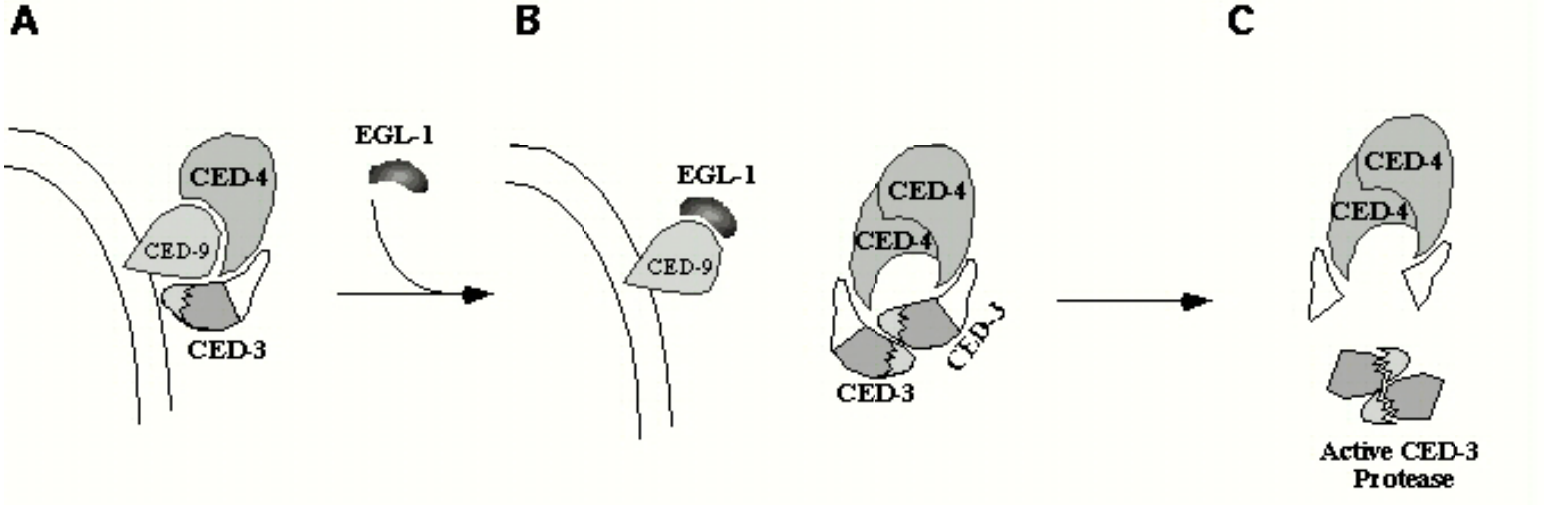
ced-5
ced-10
nuc-1


A
B
C
CED-4
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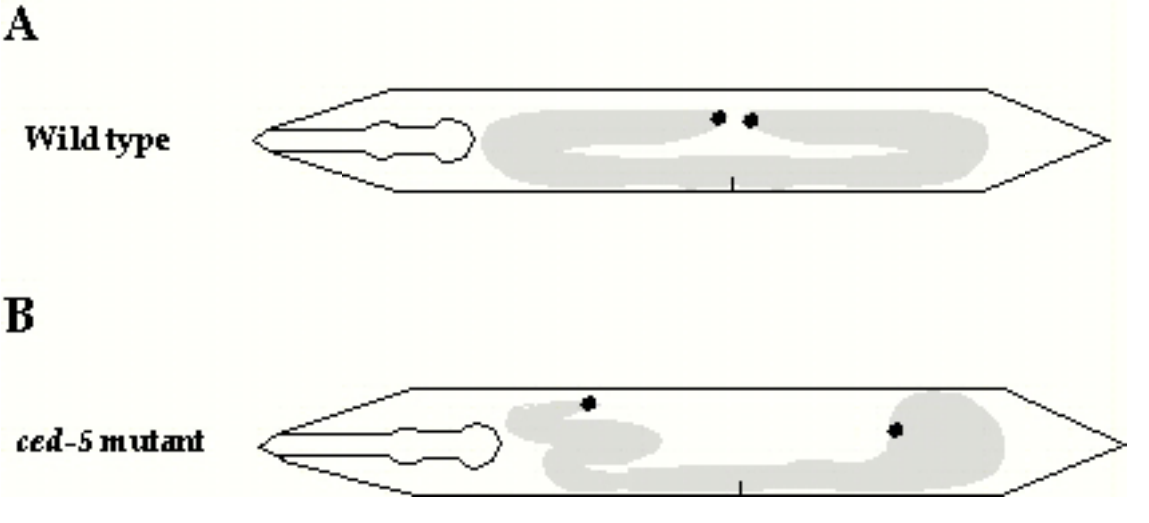
Active CED-3
Protease


A
Wild type
B
ced-5 mutant